33rd Annual International Conference of the IEEE EMBS
Boston, Massachusetts USA, August 30 - September 3, 2011

Mitochondrial Membrane Permeabilization with Nanosecond Electric Pulses

P. Thomas Vernier, *Senior Member, IEEE*

***Abstract*— Ultra-short, high-field electric pulses permeabilize plasma and intracellular membranes. We report here nanosecond pulse-induced permeabilization of mitochondrial membranes in living cells. Using four independent methods based on fluorescent dyes — JC-1, rhodamine 123, tetramethyl rhodamine ethyl ester, and cobalt-quenched calcein — we show that as few as five, 4 ns, 10 MV/m pulses delivered at 1 kHz cause an increase of the inner mitochondrial membrane permeability and an associated loss of mitochondrial membrane potential. The most likely interpretation of these results is a pulse-induced permeabilization of the inner mitochondrial membrane.**

I. Introduction

Nanosecond, megavolt-per-meter pulsed electric fields not only permeabilize the plasma membrane [1], but also nondestructively perturb the intracellular environment, causing calcium bursts [2,3], eosinophil sparklers [4], vacuole permeabilization [5,6], and the appearance of apoptotic indicators such as release of cytochrome c into the cytoplasm [7] and caspase activation [8,9]. To test the theoretical prediction of permeabilization of mitochondria by nanosecond electric pulses [10], which might then be followed by release of apoptosis-inducing factors into the cytoplasm, we investigated the effects of nanoelectropulses (4 ns, 10 MV/m) on mitochondrial membrane permeability and mitochondrial membrane potential by monitoring JC-1 [11], rhodamine 123 (R123), tetramethyl rhodamine ethyl ester (TMRE) [12, 13], and cobalt-quenched calcein [14] fluorescence after nanoelectropulse exposure. We also measured plasma membrane permeabilization as indicated by influx of YO-PRO-1 and propidium iodide (PI) [1]. Our results are consistent with pulse-induced permeabilization of mitochondrial membranes and an associated loss of mitochondrial membrane potential. At lower pulse doses we see a reduction in mitochondrial membrane potential with minimal plasma membrane permeabilization, suggesting that under some conditions significant intracellular membrane permeabilization can be achieved without significant permeabilization of the external cell membrane.

Manuscript received March 26, 2011. This work was supported in part by the U.S. Air Force Office of Scientific Research.

P. T. Vernier is with the Ming Hsieh Department of Electrical Engineering and MOSIS, Information Science Institute, in the Viterbi School of Engineering, University of Southern California, Los Angeles, CA 90089 USA (phone: 310-448-8752; fax: 310-823-5624; e-mail: vernier@usc.edu).

II. Materials and Methods

A. Cell Culture

Jurkat T lymphoblasts (ATCC TIB-152) were grown in RPMI 1640 (Mediatech) containing 10% heat-inactivated fetal bovine serum (Gibco), 2 mM L-glutamine (Gibco), 50 units/mL penicillin (Gibco), and 50 μg/mL streptomycin (Gibco). Cells were cultured at 37 °C in a humidified, 5% CO_2 atmosphere and concentrated to 2 x 10^7 cells/mL for pulse treatment.

B. Pulsed Electric Field Exposures

For microscopic observation, cells were placed in a microchamber 100 μm wide, 30 μm deep, and 15 mm long, with platinum electrode walls on a glass microscope slide. A resonant-charged, solid-state Marx bank-driven, hybrid-core compression, diode-opening switch pulse generator designed and assembled at the University of Southern California [15] delivered 4 ns, 10 MV/m electrical pulses at a 1 kHz repetition rate to the microchamber electrodes mounted on the microscope stage in ambient atmosphere at room temperature. For JC-1 experiments cells were exposed to 20 ns, 2 MV/m pulses in electroporation cuvettes [9].

C. Fluorescence Microscopy

Observations of live cells during and after pulse exposure were made with a Zeiss Axiovert 200 epifluorescence microscope with 63X water immersion objective and Hamamatsu ImageEM EM-CCD camera. Captured images were analyzed with Hamamatsu SimplePCI software. For JC-1 experiments cells were analyzed with flow cytometry, as previously described [9].

1. *Cobalt-quenched calcein*. After loading of cells with calcein-AM (Molecular Probes), mitochondria can be identified by the cobalt quenching of cytoplasmic but not mitochondrial calcein fluorescence (cobalt ions are taken up by cells but do not readily pass through the mitochondrial membrane) [14,16]. Cells were loaded with 500 nM calcein-AM in the presence of 1 mM $CoCl_2$ at 37 °C in a 5% CO_2 atmosphere for 20 minutes. Before pulse treatment, cells were washed and resuspended in fresh RPMI 1640 with or without 1 mM Co^{2+} in the medium.

2. *Mitochondrial transmembrane potential-sensitive fluorochromes JC-1 [11], rhodamine 123 (R123) and tetramethyl rhodamine ethyl ester (TMRE) [12,17,18]*. Cells were incubated with 750 nM JC-1 (Invitrogen, Molecular Probes), 1 μM R123 (Invitrogen, Molecular Probes) for 45

minutes or 50 nM TMRE (Invitrogen, Molecular Probes) for 20 minutes. After incubation, cells were centrifuged and resuspended in fresh RPMI 1640 for pulse exposure. For a positive control, the proton ionophore uncoupler of oxidative respiration carbonyl cyanide 3-chlorophenylhydrazone (CCCP) was added to the cell suspension at 20 µM [19].

3. *MitoTrackers*. MitoTracker Green and MitoTracker Orange (Invitrogen, Molecular Probes) were used to confirm the labeling of mitochondria. Cell loading was performed according to the manufacturer's protocol (100 nM MitoTracker Orange with cobalt-quenched calcein and R123 and 50 nM MitoTracker Green with TMRE).

4. *Plasma membrane integrity*. Intracellular YO-PRO-1 (Invitrogen, Molecular Probes) and propidium iodide (PI; Invitrogen, Molecular Probes) fluorescence were used as indicators of plasma membrane permeabilization as described previously [1]. Cells were suspended in RPMI 1640 with 10 µM YO-PRO-1 or 7.5 µM PI just before pulse delivery.

III. Results

A. 5-Nanosecond Electric Pulses Cause Loss of Mitochondrial Membrane Potential

The green:red ratio of intracellular JC-1 fluorescence is increased (Fig. 1), and the intracellular fluorescence of R123 and TMRE is significantly reduced (Fig. 2) following nanoelectropulse exposure, indicating a loss of mitochondrial membrane potential. The extent of pulse-induced mitochondrial membrane permeabilization depends on the number of pulses — more pulses cause a greater increase in the JC-1 green:red fluorescence ratio and a greater reduction in R123 and TMRE fluorescence.

B. Mitochondrial Depolarization Is Detected At Lower Pulse Counts Than Plasma Membrane Permeabilization

Fluorescence of calcein-stained, cobalt-quenched cells *without* cobalt ions in the external medium decreased after exposure to 5, 10 and 20 pulses, indicating mitochondrial membrane permeabilization (data not shown). No fluorescence decrease was observed after exposure to 30 pulses or more under these conditions. We interpret this to mean that plasma membrane permeabilization at these higher pulse numbers allows cobalt ions to leak out of the cells, dequenching the calcein fluorescence. On the other hand, fluorescence of calcein-stained, cobalt-quenched cells in medium *containing* cobalt ions decreased significantly after the application of 20 pulses and decreased further at higher pulse numbers (30 and 100). Under these conditions cobalt ions in the external medium allow calcein quenching even after permeabilization of the plasma membrane, since the Co^{2+} concentration gradient favors an inward flux of cobalt ions rather than a decrease in cytoplasmic Co^{2+}.

YO-PRO-1 influx, an indicator of plasma membrane permeabilization, is detectable even with five, 5 ns pulses, with significantly increased dye uptake at 20 and more pulses (data not shown). Although PI is a less sensitive detector of plasma membrane permeabilization than YO-PRO-1 [1], a small but significant influx of PI was detected after applying 20 pulses or more.

IV. Discussion

The nanosecond pulsed electric field-induced decreases in fluorescence emissions of R123, TMRE, and cobalt-quenched calcein, and the increase in the JC-1 green:red fluorescence ratio reported here are consistent with the hypothesis that nanoelectropulse exposure permeabilizes the inner membrane of mitochondria, with an associated loss of mitochondrial membrane potential. We cannot say, however, whether the pulse-induced decrease in mitochondrial membrane potential results from the formation of mitochondrial membrane permeability transition pores (or other apoptosis-related pores) or from the direct electroporation of the inner mitochondrial membrane.

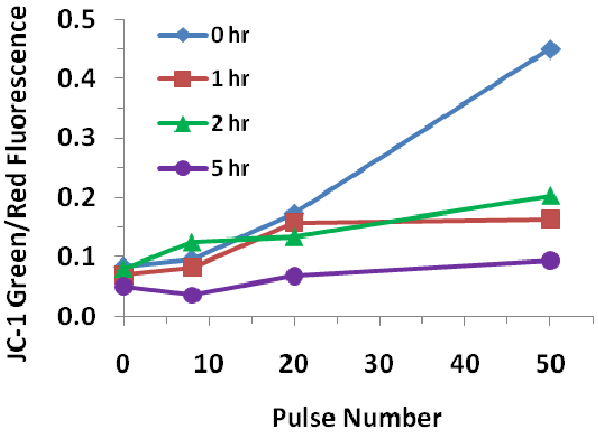

Fig. 1. Increased green:red fluorescence emission in JC-1-labeled Jurkat lymphoblasts after exposure to 20 ns, 2 MV/m electric pulses indicates loss of mitochondrial membrane potential. Cells recover within one hour.

We interpret the combination of the cobalt-quenched calcein and YO-PRO-1 and PI influx results to mean that substantial plasma membrane permeabilization occurs with pulse numbers greater than 20. This *apparent* threshold is at least in part a consequence of the detection sensitivity of the methods. We know already that YO-PRO-1 is a more sensitive indicator of plasma membrane permeabilization than PI [1,20], and we see minimal PI influx with a 20-pulse exposure under the conditions we employed in this work, while at the same time even 5 pulses result in measurable YO-PRO-1 entry into the cell. Similarly, using the cobalt-quenched calcein method for mitochondrial labeling without Co^{2+} in the medium, we observe an increase of calcein fluorescence with pulse counts above 20, suggesting a leak of intracellular Co^{2+} through the permeabilized plasma membrane, resulting in calcein dequenching. This interpretation is supported by the observation that no dequenching is observed when Co^{2+} is included in the

suspending medium at the loading (quenching) concentration.

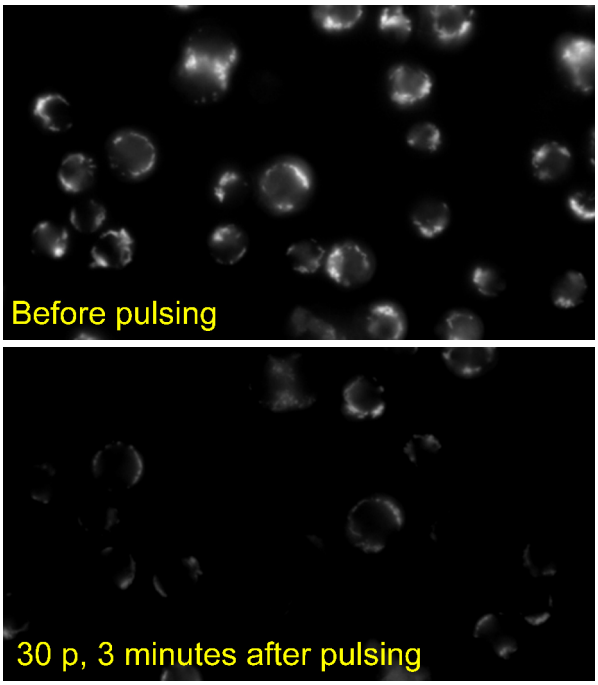

Fig. 2. Fluorescence micrographs of Jurkat T lymphoblasts showing reduction of TMRE fluorescence emission (reduction in mitochondrial membrane potential) after exposure to 30, 4 ns, 10 MV/m electric pulses.

ACKNOWLEDGMENT

Experimental expertise and helpful discussions and support from Tina Batista Napotnik, Yu-Hsuan Wu, Martin A. Gundersen, Jason Sanders, and Damijan Miklavcic are gratefully acknowledged.

REFERENCES

[1] Vernier, P. T., Y. Sun, and M. A. Gundersen. 2006. Nanoelectropulse-driven membrane perturbation and small molecule permeabilization. *BMC Cell Biol.* 7:37.

[2] Vernier, P. T., Y. Sun, L. Marcu, S. Salemi, C. M. Craft, and M. A. Gundersen. 2003. Calcium bursts induced by nanosecond electric pulses. *Biochem. Biophys. Res. Commun.* 310:286-295.

[3] White, J. A., P. F. Blackmore, K. H. Schoenbach, and S. J. Beebe. 2004. Stimulation of capacitative calcium entry in HL-60 cells by nanosecond pulsed electric fields. *J. Biol. Chem.* 279:22964-22972.

[4] Schoenbach, K. H., S. J. Beebe, and E. S. Buescher. 2001. Intracellular effect of ultrashort electrical pulses. *Bioelectromagnetics* 22:440-448.

[5] Tekle, E., H. Oubrahim, S. M. Dzekunov, J. F. Kolb, K. H. Schoenbach, and P. B. Chock. 2005. Selective field effects on intracellular vacuoles and vesicle membranes with nanosecond electric pulses. *Biophys. J.* 89:274-284.

[6] Napotnik, T. B., M. Rebersek, T. Kotnik, E. Lebrasseur, G. Cabodevila, and D. Miklavcic. 2010. Electropermeabilization of endocytotic vesicles in B16 F1 mouse melanoma cells. *Med Biol Eng Comput* 48:407-413.

[7] Beebe, S. J., P. M. Fox, L. J. Rec, K. Somers, R. H. Stark, and K. H. Schoenbach. 2002. Nanosecond pulsed electric field (nsPEF) effects on cells and tissues: Apoptosis induction and tumor growth inhibition. *IEEE Trans. Plasma Sci.* 30:286-292.

[8] Beebe, S. J., P. M. Fox, L. J. Rec, E. L. Willis, and K. H. Schoenbach. 2003. Nanosecond, high-intensity pulsed electric fields induce apoptosis in human cells. *FASEB J.* 17:1493-1495.

[9] Vernier, P. T., A. M. Li, L. Marcu, C. M. Craft, and M. A. Gundersen. 2003. Ultrashort pulsed electric fields induce membrane phospholipid translocation and caspase activation: Differential sensitivities of Jurkat T lymphoblasts and rat glioma C6 cells. *IEEE Trans Dielect Elect Ins* 10:795-809.

[10] Kotnik, T., and D. Miklavcic. 2006. Theoretical evaluation of voltage inducement on internal membranes of biological cells exposed to electric fields. *Biophys. J.* 90:480-491.

[11] Cossarizza, A., and S. Salvioli. 2001. Analysis of mitochondria during cell death. *Methods Cell Biol.* 63:467-486.

[12] Emaus, R. K., R. Grunwald, and J. J. Lemasters. 1986. Rhodamine 123 as a probe of transmembrane potential in isolated rat-liver mitochondria: spectral and metabolic properties. *Biochim. Biophys. Acta* 850:436-448.

[13] Ichas, F., and J. P. Mazat. 1998. From calcium signaling to cell death: two conformations for the mitochondrial permeability transition pore. Switching from low- to high-conductance state. *Biochim. Biophys. Acta* 1366:33-50.

[14] Petronilli, V., G. Miotto, M. Canton, M. Brini, R. Colonna, P. Bernardi, and F. Di Lisa. 1999. Transient and long-lasting openings of the mitochondrial permeability transition pore can be monitored directly in intact cells by changes in mitochondrial calcein fluorescence. *Biophys. J.* 76:725-734.

[15] Sanders, J. M., A. Kuthi, Y. H. Wu, P. T. Vernier, and M. A. Gundersen. 2009. A linear, single-stage, nanosecond pulse generator for delivering intense electric fields to biological loads. *IEEE Trans Dielect Elect Ins* 16:1048-1054.

[16] Huser, J., C. E. Rechenmacher, and L. A. Blatter. 1998. Imaging the permeability pore transition in single mitochondria. *Biophys. J.* 74:2129-2137.

[17] Chen, L. B. 1988. Mitochondrial membrane potential in living cells. *Annu. Rev. Cell Biol.* 4:155-181.

[18] Scaduto, R. C., Jr., and L. W. Grotyohann. 1999. Measurement of mitochondrial membrane potential using fluorescent rhodamine derivatives. *Biophys. J.* 76:469-477.

[19] Bortner, C. D., and J. A. Cidlowski. 1999. Caspase independent/dependent regulation of K^+, cell shrinkage, and mitochondrial membrane potential during lymphocyte apoptosis. *J. Biol. Chem.* 274:21953-21962.

[20] Bowman, A. M., O. M. Nesin, O. N. Pakhomova, and A. G. Pakhomov. 2010. Analysis of plasma membrane integrity by fluorescent detection of Tl^+ uptake. *J. Membr. Biol.* 236:15-26.